

The Autophagosomal SNARE Protein Syntaxin 17 Is an Essential Factor for the Hepatitis C Virus Life Cycle

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ABSTRACT

Syntaxin 17 is an autophagosomal SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein required for the fusion of autophagosomes with lysosomes to form autolysosomes and thereby to deliver the enclosed contents for degradation. Hepatitis C virus (HCV) induces autophagy. In light of the observation that the number of viral particles formed by HCV-infected cells is much greater than the number of infectious viral particles finally released by HCV-infected cells, the regulation of fusion between autophagosomes and lysosomes might fulfill a key function controlling the number of released virions. HCV-replicating cells possess a decreased amount of syntaxin 17 due to impaired expression and increased turnover of syntaxin 17. Overexpression of syntaxin 17 in HCV-replicating cells diminishes the number of released infectious viral particles and decreases the amount of intracellular retained viral particles by favoring the formation of autolysosomes, in which HCV particles are degraded. Inhibition of lysosomal acidification by bafilomycin rescues the decreased release of virions from syntaxin 17-overexpressing cells, while induction of autophagy by rapamycin enforces the impairment of release under these conditions. Vice versa, inhibition of syntaxin 17 expression by specific small interfering RNAs results in an elevated amount of intracellular retained viral particles and facilitates the release of HCV virions by impairment of autophagosome-lysosome fusion. HCV genome replication, however, is not affected by modulation of syntaxin 17 expression. These data identify syntaxin 17 to be a novel factor controlling the release of HCV. This is achieved by regulation of autophagosome-lysosome fusion, which affects the equilibrium between the release of infectious viral particles and lysosomal degradation of intracellular retained viral particles.

IMPORTANCE

Hepatitis C virus (HCV) induces autophagy. Syntaxin 17 is an autophagosomal SNARE protein required for the fusion of autophagosomes with lysosomes. In HCV-infected cells, a major fraction of the *de novo*-synthesized viral particles is not released but is intracellularly degraded. In this context, the effect of HCV on the amount and distribution of syntaxin 17 and the relevance of syntaxin 17 for the viral life cycle were investigated. This study demonstrates that the amount of syntaxin 17 decreased in HCV-replicating cells. In addition, syntaxin 17 is identified to be a novel factor controlling the release of HCV, and the relevance of autophagosome-lysosome fusion as a regulator of the amount of released viral particles is revealed. Taken together, these findings indicate that syntaxin 17 is involved in the regulation of autophagosome-lysosome fusion and thereby affects the equilibrium between the release of infectious viral particles and the lysosomal degradation of intracellularly retained viral particles.

1 Seventy million people worldwide are chronically infected with hepatitis C virus (HCV). Chronic HCV infection is associated with an elevated risk of developing liver cirrhosis and hepatocellular carcinoma (1). HCV is a single-stranded, positive-sense RNA virus that belongs to the *Flaviviridae* family. The viral RNA encodes a single polyprotein of about 3,100 amino acids, which is cleaved co- and posttranslationally by cellular and viral proteases into 10 viral proteins: the structural proteins (core, E1, E2), which build up the viral particles; the p7 polypeptide, which forms an ion channel; and the nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), which support viral replication and assembly processes (2–5).

NS5A is known to act as a regulator of intracellular signal transduction cascades. The kinase c-Raf was identified to bind to the C-terminal domain of NS5A. Thereby, c-Raf is located at the HCV replication complex (6, 7). The interaction of NS5A with c-Raf leads to the activation of c-Raf in association with the phosphorylation of c-Raf at serine 338 (6, 7). Moreover, it was found that inhibition of c-Raf blocks HCV replication (6, 8). However, due to the delocalization of c-Raf to the replicon complex/endoplasmic reticulum (ER) membrane, c-Raf in HCV-replicating cells is

withdrawn from the classic MEK/extracellular signal-regulated kinase (ERK) signaling pathway (6). Therefore, although c-Raf is activated in HCV-replicating cells, signal transduction to the MEK/ERK pathway is impaired.

The HCV infection cycle is tightly associated with lipid metabolism. HCV replication occurs on the cytoplasmic face of the ER in the replication complexes (RCs) formed by nonstructural proteins. HCV replication and morphogenesis take place at specialized rearranged intracellular ER membranes, the so-called membranous web, that are enriched in proteins involved

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in very-low-density lipoprotein (VLDL) assembly (9–11). Viral morphogenesis starts at the surface of lipid droplets (LDs). The viral core protein is directed to LDs via diacylglyceroltransferase-1 (DGAT1) and recruits the other viral proteins and the RCs to LD-associated membranes (12, 13). The nonstructural protein NS5A has an RNA binding domain and plays an essential role for the transfer of viral genomes from the RCs to the surface of LDs (14). The sorting factor TIP47 that binds to NS5A was described to target the NS5A-RNA complex to LDs. Moreover, TIP47, which has an *apoE*-like domain and binds to lipoproteins, was found to be associated with the lipo-viro-particle (LVP). However, if the Rab9 binding site in TIP47 is destroyed, TIP47 still binds to the viral particle but the complex is trapped in the autophagosomal-lysosomal compartment (15). Only a small fraction of the *de novo*-synthesized structural proteins/viral particles is indeed released as infectious viral particles, while the majority of the *de novo*-synthesized particles is intracellularly retained and degraded (16, 17).

Autophagy (self-eating) is a tightly regulated, conserved cellular process for the final degradation of intracellular components in lysosomes. Autophagy has multiple functions in many physiological processes, such as stress responses, cellular quality control, and cellular homeostasis maintenance (18). The mature autophagosomes can directly fuse with lysosomes to form autolysosomes and to deliver the enclosed contents for degradation (19). Syntaxin 17 (Stx17) is an autophagosomal SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein, and it is required for the fusion of autophagosomes with endosomes/lysosomes by interaction with SNAP29 and the autophagosomal SNARE VAMP8 at a late step of autophagy (20, 21). Syntaxin 17 is stored in the cytosol until it is recruited to the outer membrane of completed autophagosomes upon their induction (21).

A variety of reports have described that HCV, like other pathogens, hijacks the autophagic pathway for viral morphogenesis and the release and removal of nonreleased viral particles (22, 23). Knockdown of autophagy-related gene Atg7 or Beclin-1 in HCV-infected Huh7.5.1 cells decreases the production of infectious viral particles, which suggests that autophagy contributes to the effective production of HCV particles (24).

This study aims to investigate the relevance of syntaxin 17 to the HCV life cycle and, thereby, to reveal the role of autophagosome-lysosome fusion for the turnover of viral particles.

MATERIALS AND METHODS

Cell culture. The Huh7-derived cell line Huh7.5 (25), which is highly permissive for HCV RNA replication, was used for electroporation and infection experiments. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (complete DMEM). Huh7 cell clone 9-13 (Huh9-13 cells) harboring the HCV replicon I377/NS3-3' (here referred to as Huh7 I377/NS3-3'/wt/9-13) were grown in complete DMEM with 1 mg/ml G418 as described previously (26). To discriminate these cells from cells producing infectious viral particles, Huh9-13 cells were designated subgenomic replicon-expressing cells. Isolation, cultivation, and infection of primary human hepatocytes (PHHs) were performed as described previously (27, 28). The use of primary human hepatocytes was approved by the ethics committee of the Medical Faculty of the University of Frankfurt (decision 343/13). The breeding of NS5A transgenic mice and analysis of liver samples were approved by the local authority (RP Darmstadt).

Generation of HCV-replicating cells and HCV-negative control cells. HCV-replicating cells and the negative-control cells were generated by electroporation of Huh7.5 cells with JFH1, J6, or replication-deficient variant GND HCV RNA, which was generated by *in vitro* transcription. *In vitro* transcription and electroporation of HCV RNAs were performed as described in reference 29. Huh7.5 cells (4×10^6) were electroporated with 10 µg HCV RNA and cultured in a T75 flask as described above. At 3 days after electroporation, the cells were transferred into a T175 flask with fresh medium and grown for 4 days. The medium was changed every 2 days. Cells were seeded for experiments at 7 days after electroporation. At this time point, more than 85% of the cells were HCV positive (HCV-replicating cells), as evidenced by immunofluorescence microscopy using core-specific antibody.

Plasmids. Plasmids pFK-JFH1/GND, pFK-JFH1/wt, and pFK-JFH1/J6 have been described previously (30). Flag-syntaxin 17 was obtained from Addgene (21). Plasmids carrying constitutive active Raf (v-Raf) and for a transdominant negative Raf mutant (tdn-raf) (pRafC4) were described previously (31). Plasmid pHA-NS5A was described in reference 7.

Antibodies. Anti-core antibody (catalog number MA1-080) and LysoTracker (catalog number L7528) were obtained from Thermo Scientific. Anti-NS3 antibody (catalog number 8G-2) was obtained from Abcam. For detection of NS5A, polyclonal rabbit-derived serum was used (6). Anti-β-actin (catalog number AC-74) and anti-syntaxin 17 (catalog number HPA001204) antibodies were purchased from Sigma-Aldrich. Anti-p62 antibody (catalog number GP62-C) was obtained from Progen. Secondary antibodies for Western blotting were purchased from LI-COR, Inc., or GE Healthcare (peroxidase-conjugated secondary antibodies). Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) and Cy3- and Cy5-conjugated secondary antibodies (Jackson Immuno Research Laboratories, Inc.) were used for immunofluorescence staining.

Infection of primary human hepatocytes. PHHs were infected with HCV from the cell culture supernatant for 24 h. Total RNA was isolated 120 h after infection, and the HCV- and syntaxin 17-specific RNAs were quantified by real-time PCR (RT-PCR). Uninfected Huh7.5 cells served as a control.

Transient transfection and silencing. HCV-replicating cells (more than 85% of which were positive for HCV) were seeded 1 day prior to transfection or silencing. For overexpression, cells in one well (6-well plate) were transfected with 1 µg the syntaxin 17-expressing construct (pFlag-syntaxin 17) using linear polyethyleneimine (PEI; Polysciences, Inc.) as described in reference 32. For silencing, 20 nM syntaxin 17-specific small interfering RNA (siRNA; Santa Cruz) was transfected using an N-TER nanoparticle siRNA transfection system (Sigma) according to the manufacturer's protocol. At 72 h after transfection, the supernatant was collected and used for HCV genome quantification and virus titration. Meanwhile, gene expression was analyzed by Western blotting, RT-PCR, and immunofluorescence.

SDS-PAGE and Western blot analyses. SDS-PAGE and Western blot analyses were performed as described in reference 6. Proteins were detected by chemiluminescence and exposed to Hyperfilm ECL autoradiography film. The signal intensities from each blot were quantified by the use of Image studio software after densitometric scanning of the films. The graphs in the figures show the quantity of the target protein signal in reference to that of β-actin. The quantities are based on those from three independent experiments. The blots of one representative experiment are shown below.

Indirect immunofluorescence analyses. Indirect immunofluorescence analyses were performed as described in reference 15. In the case of staining with LysoTracker, the cells were incubated with LysoTracker stain diluted in cell culture medium for 30 min before the cells were fixed with 4% formaldehyde and stained. Immunofluorescence staining was analyzed by use of a confocal laser scanning microscope (CLSM; CLSM 510 Meta, Carl Zeiss) and ZEN 2009 software. The objectives used were 40× (numerical aperture [NA], 1.3) and 100× (NA, 1.46). The fluores-

cence intensity in the whole area shown in the images was quantified with ZEN 2009 software (Zeiss).

Tissue samples. Tissue samples were analyzed as described in reference 33.

RT-PCR. Total RNA isolation and cDNA synthesis were performed as described in reference 15. RT-PCR was performed as described in reference 34 with the following primers: R6-260-R19(HCV fw) (5'-ATGACC ACAAGGCCCTTCG-3'), R6-130-146(HCV rev) (5'-CGGGAGAGCCA TAGTGG-3'), syntaxin 17 fw (5'-GAGAAATTGAGAACTTTGTTTG-3'), syntaxin 17 rev (5'-AATGGAGTTGGAGAAATTCTGC-3'), GAPDH fw (5'-GACCCCTTCATTGACCTCAAC-3'), and GAPDH rev (5'-TGG ACTGTGGTCATGAGTCC-3'). The amount of specific transcripts was normalized to the amount of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA.

Autophagy modulation. At 48 h after transfection, cells were treated with 100 nM bafilomycin A1 (Sigma) for 16 h to inhibit lysosomal acidification or were treated with 100 nM rapamycin (Selleck Chemicals) for 16 h to induce autophagy.

Virus titration. Virus titers were determined from the 50% tissue culture infective dose (TCID₅₀) as described previously (35). For the supernatant derived from the autophagy modulation experiment, 0.025- μ m-pore-size MF-Millipore membrane filters were used to get rid of bafilomycin or rapamycin. For determination of the number of intracellular TCID₅₀s, the cells were scratched down and lysed by freeze-thawing 4 times with liquid nitrogen and a water bath. After 10 min of centrifugation at full speed, the supernatant was used for infection with the TCID₅₀. For detection of HCV-positive cells, the NS5A-specific serum described previously (6) was used.

Quantification of released viral genomes. A QIAamp DSP virus kit was used for the isolation and purification of viral nucleic acids from the cell culture supernatant according to the manufacturer's instruction. The isolated HCV genomes were quantified with an Artus HCV RG RT-PCR kit on a Rotor-Gene Q instrument according to the manufacturer's protocol.

Statistical analyses. Results are described as means \pm standard errors of the means (SEMs). The significance of the results was analyzed by a ratio *t* test using GraphPad Prism software. The bars in the figures represent SEMs.

In bar graphs showing a change compared to the result for the control, the value for the control group was arbitrarily set equal to 1. Here, a standard deviation for the control group cannot be reported, as standardization of the measured values (relative to the control group) was performed for each of the independent assays. Therefore, measurements for the treatment groups in each assay were dependent (matched).

RESULTS

Reduced amount of syntaxin 17 in HCV-positive cells. In order to study the effect of HCV on the intracellular level of syntaxin 17, Western blot analyses of cellular lysates derived from HCV-replicating cells (JFH1 and J6) and the corresponding control cells (GND) were performed. The blots show that the amount of syntaxin 17 was significantly decreased in HCV-positive cells (Fig. 1a). This was confirmed by immunofluorescence microscopy analyses of HCV-replicating cells (J6) and HCV-negative control cells (GND) (Fig. 1b). To study the variation in the syntaxin 17 level over time, the amounts of syntaxin 17 and HCV core were analyzed by Western blotting over 102 h after electroporation of Huh7.5 cells with the J6 genome. The amount of syntaxin 17 started to decrease after 6 h. At 42 h after electroporation, a constant decreased level was reached (Fig. 1c). Moreover, immunofluorescence microscopy of liver tissue samples derived from HCV-infected patients further confirmed the reduced amount of syntaxin 17 by comparison to that in liver tissue samples from HCV-negative patients (Fig. 1d).

To study the effect of HCV genome replication on the amount of syntaxin 17 in the absence of virus morphogenesis, cells expressing HCV subgenomic replicon (Huh7 I377/NS3-30/wt/9-13) (26) were used and the amount of syntaxin 17 in cells expressing the HCV subgenomic replicon was compared to that in Huh7 cells. Western blot and immunofluorescence microscopy analyses showed a significantly decreased amount of syntaxin 17 in cells expressing the HCV subgenomic replicon (Huh9-13 cells) compared with that in control Huh7 cells (Fig. 1e and f).

These data demonstrate a reduced amount of syntaxin 17 in cells replicating either a subgenomic replicon or a complete HCV genome that enables the morphogenesis/release of infectious viral particles.

Reduced expression and increased turnover due to the HCV-dependent induction of autophagy lead to diminished amounts of syntaxin 17 in HCV-replicating cells. To investigate whether the reduced intracellular amount of syntaxin 17 in HCV-replicating cells is due to decreased gene expression, RNA was isolated from HCV-replicating and HCV-negative control cells, and the amount of syntaxin 17-specific mRNA was quantified by RT-PCR. The RT-PCR analyses showed a decreased level of syntaxin 17-specific transcripts in HCV-positive cells (Fig. 2a). To study the variation in the level of syntaxin 17 expression over time, the amounts of syntaxin 17-specific transcripts and HCV genomes were analyzed by RT-PCR over 102 h after electroporation of Huh7.5 cells with the J6 genome. The amount of syntaxin 17-specific transcripts started to decrease after 6 h, while the level of HCV-specific RNA increased (Fig. 2b).

To confirm the inhibitory effect of HCV on syntaxin 17 expression, primary human hepatocytes (PHHs) were infected with HCV from the cell culture supernatant and the level of syntaxin 17 and HCV expression was analyzed by RT-PCR. Results comparable to those obtained with HCV-replicating Huh7.5 cells were obtained with HCV-infected PHHs (Fig. 2c). These data demonstrate that HCV represses the expression of syntaxin 17.

HCV was described to impair c-Raf/MEK/ERK signaling (6). NS5A binds c-Raf and thereby delocalizes c-Raf to the replicon complex and withdraws c-Raf from the MEK/ERK signaling cascade (6). To study whether impaired signal transduction via c-Raf/MEK/ERK is causative for the reduced expression of syntaxin 17 in HCV-replicating cells, c-Raf signaling was modulated in Huh7.5 cells by overexpression of a transdominant negative mutant (RafC4). A reduced level of syntaxin 17-specific transcripts was observed when c-Raf signaling was inhibited by expression of RafC4 (Fig. 2d). Previous data provided evidence that NS5A interrupts c-Raf signaling (6, 7, 36). According to this, it was further investigated whether the HCV-dependent repression of syntaxin 17 transcription is due to an NS5A-mediated interruption of c-Raf signaling. Indeed, a decreased number of syntaxin 17-specific transcripts was found in Huh7.5 cells that were transfected with a NS5A expression vector compared to the number found in control transfected cells (Fig. 2e) and a decreased amount of syntaxin 17 was found in liver tissue samples derived from transgenic mice that express NS5A under the control of the albumin promoter (36) (Fig. 2f).

The decreased expression of syntaxin 17 in HCV-replicating cells could be rescued by transfection with a constitutive active mutant of Raf (v-Raf) or by stimulation with phorbol myristate acetate (PMA), as shown by quantification of syntaxin 17-spe-

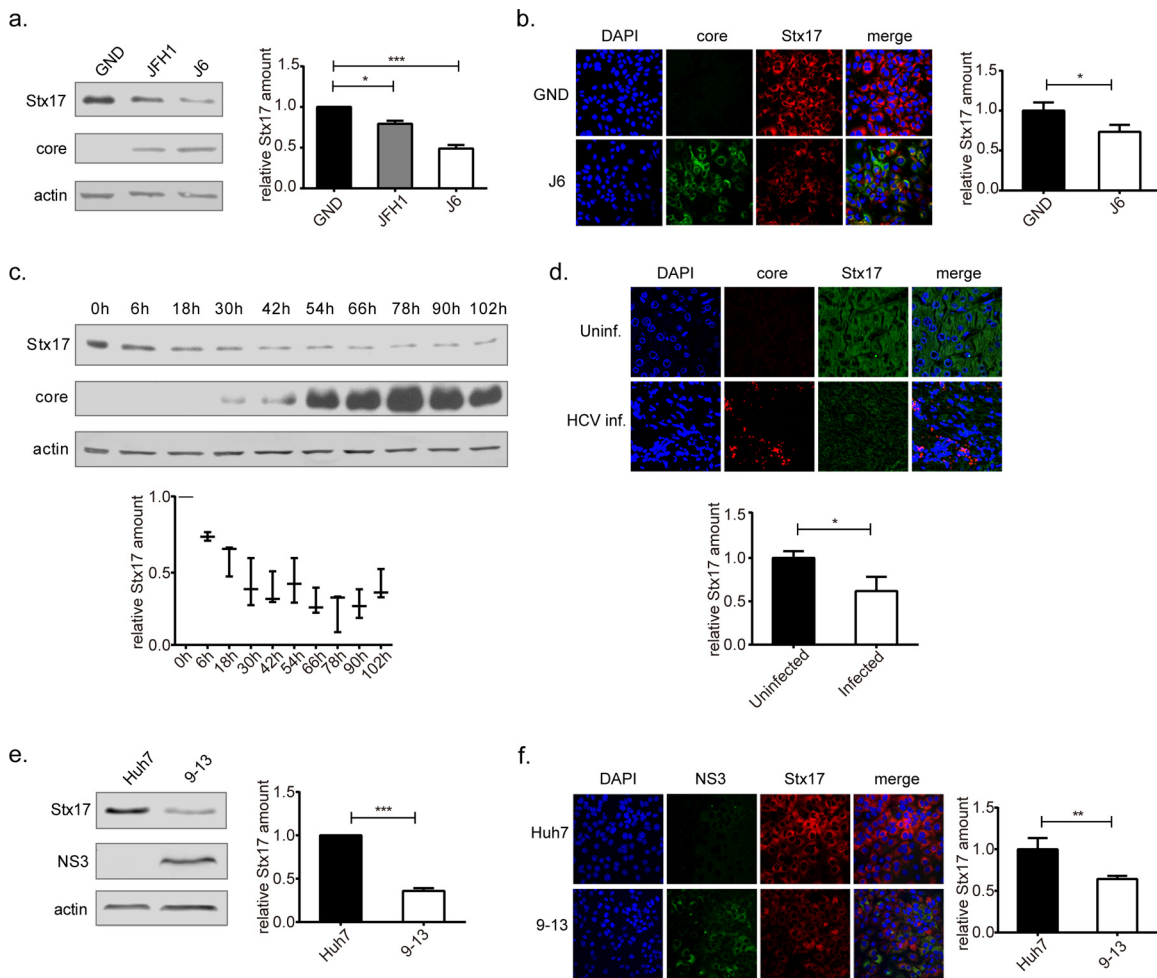


FIG 1 Reduced amount of syntaxin 17 in HCV-positive cells. (a) (Left) Western blot analyses of cellular lysates derived from HCV-replicating cells (JFH1 and J6) and HCV-negative cells (GND). The experiment was performed in triplicate. One representative blot is shown. (Right) Results of quantification by densitometric scanning from three independent experiments. (b) (Left) CLSM analyses of HCV-replicating cells (J6) and HCV-negative cells (GND). The cells were stained with core-specific antibody (green) and syntaxin 17-specific antibody (red). The nuclei were stained with DAPI (4',6-diamidino-2-phenylindole; blue). Laser intensity and digital gain were kept constant for GND and J6 cells. Magnifications, $\times 40$. (Right) Results of quantification of the fluorescence intensity of syntaxin 17 obtained with ZEN 2009 software. (c) (Top) Western blot analyses of cellular lysates derived from J6 cells at different time points after electroporation. The experiment was performed in triplicate. One representative blot is shown. (Bottom) Results of quantification of syntaxin 17 by densitometric scanning. (d) (Top) CLSM analyses of liver tissue samples derived from HCV-infected patients and HCV-negative patients. Four uninfected and four HCV-positive tissue samples were analyzed, and the results for one of them are shown. Syntaxin 17 was visualized by green fluorescence, and core was visualized by red fluorescence. Nuclei were stained with DAPI (blue). Laser intensity and digital gain were adjusted for one reference sample and kept constant in all samples. (Bottom) Results of quantification of the fluorescence intensity of syntaxin 17 from all the samples. Magnifications, $\times 40$. (e) (Left) Western blot analyses of cellular lysates derived from cells harboring the HCV subgenomic replicon (Huh9-13 cells) and control Huh7 cells. (Right) Results of quantification by densitometric scanning from three independent experiments. (f) (Left) CLSM analyses of Huh9-13 cells and Huh7 cells. The cells were stained with NS3-specific antibody (green) and syntaxin 17-specific antibody (red). The nuclei were stained with DAPI (blue). (Right) Results of quantification of the fluorescence intensity of syntaxin 17. Magnifications, $\times 40$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cific transcripts by RT-PCR or Western blotting analyses (Fig. 2g and h).

Syntaxin 17 is required for fusion of autophagosomes and lysosomes at a late step of autophagy. Since it is well established that HCV induces autophagy (22–24), it was investigated whether the HCV-dependent induction of autophagy is an additional factor contributing to the reduced amount of syntaxin 17 in HCV-replicating cells. For this purpose, a late step in the autophagy process, the fusion between autophagosomes and lysosomes, was inhibited by bafilomycin in HCV-replicating cells and in the corresponding HCV-negative cells. Western blot analyses revealed that bafilomycin treatment of HCV-negative cells slightly increased the amount

of syntaxin 17 compared to that in the untreated cells (Fig. 2i). In contrast to this, the relative increase in the amount of syntaxin 17 in bafilomycin-treated HCV-replicating cells was more pronounced than that in HCV-negative cells (Fig. 2i and j). This indicates that HCV-dependent induction of autophagy is a further factor contributing to the reduced amount of syntaxin 17. Although bafilomycin treatment of HCV-positive cells significantly increased the amount of syntaxin 17 compared to that in the untreated HCV-replicating cells, the amount of syntaxin 17 did not reach the level measured in untreated HCV-negative cells (Fig. 2i). This confirms that the HCV-dependent induction of autophagy and impaired expression of syntaxin 17 are two factors that lead to

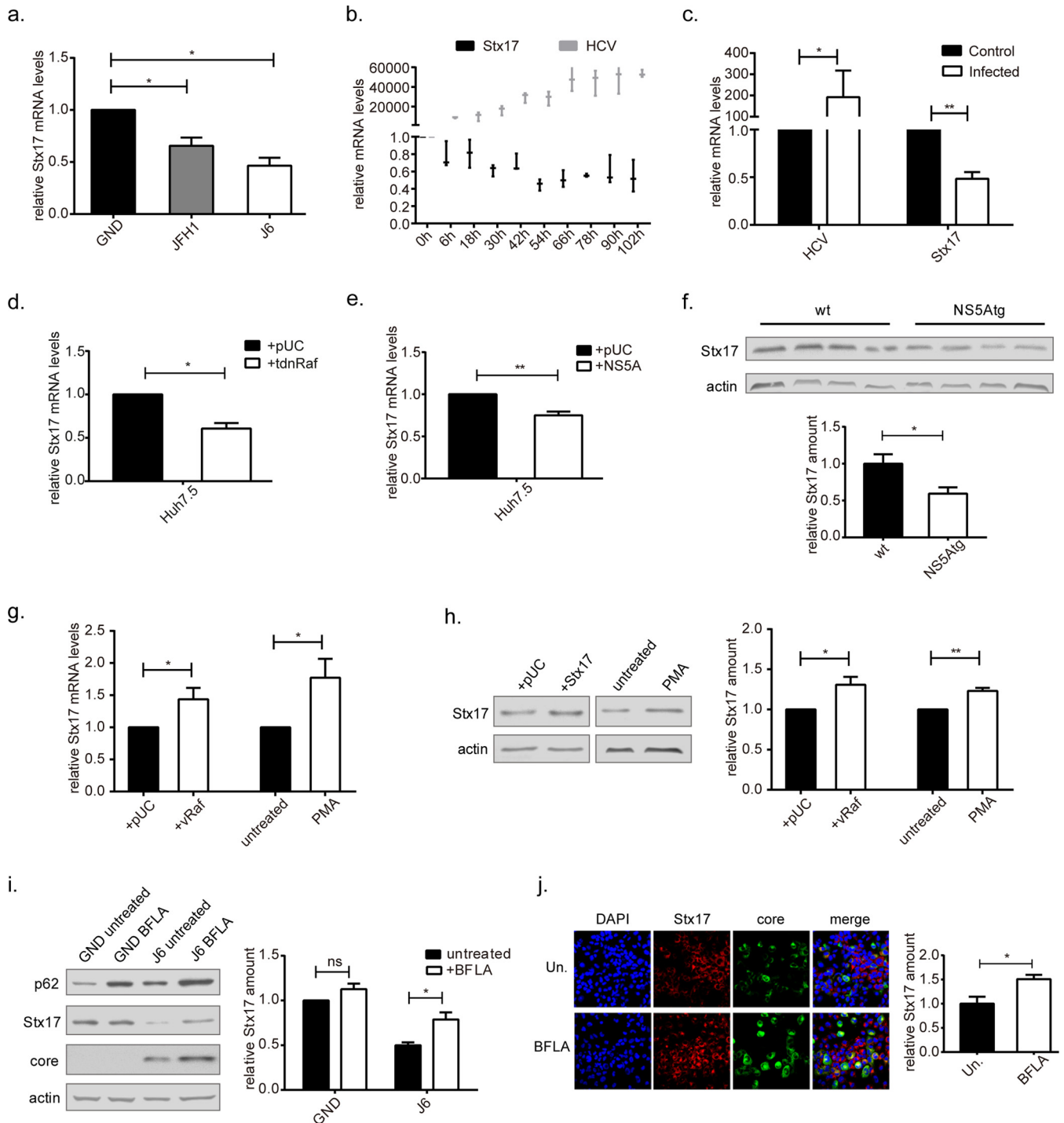


FIG 2 Reduced expression and increased turnover due to HCV-dependent induction of autophagy lead to diminished amounts of syntaxin 17 in HCV-replicating cells. (a) Quantification by RT-PCR of syntaxin 17-specific transcripts in HCV-replicating cells (JFH1 and J6) and control cells (GND). The graph shows relative values from three independent experiments. The bars represent the standard errors of the means. (b) Quantification by RT-PCR of syntaxin 17-specific and HCV-specific RNA levels in J6 cells at different time points after electroporation. The graph shows relative values from three independent experiments. (c) Quantification by RT-PCR of syntaxin 17-specific and HCV-specific transcripts in HCV-infected PHHs. The graph shows relative values from three independent experiments. (d) Quantification by RT-PCR of syntaxin 17-specific transcripts in tdn-raf (RafC4)- or pUC19-transfected Huh7.5 cells. The graph shows relative values from three independent experiments. (e) Quantification by RT-PCR of syntaxin 17-specific transcripts in pHA-NS5A- or pUC19-transfected Huh7.5 cells. The graph shows relative values from three independent experiments. (f) (Top) Western blot analyses of liver cell lysates derived from four different NS5A transgenic (NS5Atg) mice and the corresponding wild-type (wt) mice. (Bottom) Results of quantification by densitometric scanning from nine NS5A transgenic mice and nine wild-type mice. (g) Quantification by RT-PCR of syntaxin 17-specific transcripts in v-Raf (pv-Raf)-transfected or PMA-stimulated HCV-replicating cells (J6). The graph shows relative values from three independent experiments. (h) (Left) Western blot analyses of cellular lysates derived from v-Raf (pv-Raf)-transfected or PMA-stimulated HCV-replicating cells (J6) (Right) Results of quantification by densitometric

the reduced amount of syntaxin 17 in HCV-replicating cells. Moreover, there was an increased intracellular amount of core in HCV-replicating cells after bafilomycin treatment, reflecting that a fraction of core is permanently degraded by the autophagosomal/lysosomal system. If the fusion between autophagosomes and lysosomes is blocked, core accumulates, leading to an elevated amount of core in bafilomycin-treated cells.

Taken together, these data indicate that HCV reduces the amount of syntaxin 17, on the one hand, by NS5A-mediated interruption of c-Raf signaling, which affects syntaxin 17 expression, and, on the other hand, by the HCV-dependent induction of autophagy.

Overexpression of syntaxin 17 reduces the number of released HCV particles. To study the relevance of syntaxin 17 for the viral life cycle, HCV-replicating cells were transfected with a syntaxin 17 expression construct (pFlag-syntaxin 17) or the control construct pUC19. Western blot analyses of cellular lysates revealed that overexpression of syntaxin 17 leads to a decreased amount of core protein and has no significant effect on NS3 (Fig. 3a). This was confirmed by immunofluorescence microscopy: in syntaxin 17-overproducing HCV-positive cells, a reduced amount of core was found, but no obvious change in the amount or the subcellular distribution of NS3 was observed (Fig. 3b). Moreover, overexpression of syntaxin 17 reduced the amount of p62 (Fig. 3a), which is an autophagy marker (37). This reflects the induction of autophagy by overexpression of syntaxin 17.

To study the impact of syntaxin 17 overexpression on HCV particles, the intracellular and extracellular amounts of infectious viral particles from syntaxin 17- or pUC19-transfected HCV-replicating cells were analyzed by determination of the TCID₅₀. These data show that overexpression of syntaxin 17 significantly decreases the intracellular amount of viral particles and also reduces the release of infectious viral particles (Fig. 3c and d). This was confirmed by quantification of intra- and extracellular viral genomes using RT-PCR (Fig. 3e and f).

To study whether the decreased amount of released viral particles is due to an effect of syntaxin 17 overexpression on viral replication, the effect of syntaxin 17 overexpression on HCV replication was analyzed in the absence of virus morphogenesis/release. For this purpose, cells expressing the subgenomic replicon (Huh7 I377/NS3-30/wt/9-13) were again used. Western blot analyses of cellular lysates showed that overexpression of syntaxin 17 did not significantly affect the intracellular amount of NS3 and NS5A compared to that for the control (Fig. 3g). Moreover, the amount of intracellular subgenomic RNA was not significantly affected by syntaxin 17 overexpression, as determined by RT-PCR (Fig. 3h). This indicates that overexpression of syntaxin 17 does not significantly affect HCV replication.

Taken together these data indicate that overexpression of syntaxin 17 in HCV-replicating cells reduces the amount of intracellular viral particles and leads to a decreased number of released infectious viral particles but does not significantly affect genome replication.

Silencing of syntaxin 17 expression facilitates the release of viral particles. To test the hypothesis that, vice versa, decreased expression of syntaxin 17 leads to elevated levels of intracellular retained viral particles and increases the number of released HCV particles, the expression of syntaxin 17 in HCV-replicating cells was suppressed by siRNAs.

Western blot analyses of cellular lysates revealed an increased intracellular amount of core and no change in the amount of NS3 compared to the amounts in controls transfected with scrambled RNA when the expression of syntaxin 17 was blocked by specific siRNAs (Fig. 4a). This could be confirmed by confocal immunofluorescence microscopy: a decreased amount of syntaxin 17 was associated with an increased amount of core, but no obvious change in the amount of NS3 was detectable (Fig. 4b). In addition, the downregulation of syntaxin 17 expression led to an increased amount of the autophagy marker p62 (Fig. 4a).

Quantification of infectious viral particles by determination of the number of TCID₅₀s and quantification of viral genomes by RT-PCR revealed that silencing of syntaxin 17 expression leads to the accumulation of intracellular viral particles and results in increased viral release (Fig. 4c to f).

Silencing of syntaxin 17 expression in cells replicating a subgenomic replicon (Huh7 I377/NS3-30/wt/9-13) had no significant effect on the intracellular amount of NS3 and NS5A (as determined by Western blotting analyses of cellular lysates; Fig. 4g). Quantification of intracellular genomes by RT-PCR showed that the downregulation of syntaxin 17 did not affect the amount of intracellular viral genomes (Fig. 4h). This indicates that HCV genome replication is not directly affected by a decreased amount of syntaxin 17.

Taken together, these data demonstrate that silencing of syntaxin 17 expression in HCV-replicating cells increases the amount of intracellular viral particles and facilitates the release of viral particles but does not significantly affect HCV genome replication.

Neither the overexpression of syntaxin 17 nor the downregulation of syntaxin 17 expression directly affects HCV genome replication, but the number of intracellular HCV particles and the number of released viral particles are affected by modulation of syntaxin 17 expression.

Syntaxin 17 controls the amount of released viral particles by affecting the formation of autolysosomes. It is established that HCV hijacks steps of the autophagic pathway (22, 23, 38). In light of the observation that the number of viral particles formed is much greater than the number of infectious viral particles that are finally released (16, 17), regulation of the fusion between autophagosomes and lysosomes might fulfill a key function controlling the release of HCV.

Overexpression of syntaxin 17 leads to a reduced amount of p62, which is degraded by autophagy (Fig. 3a), and silencing of syntaxin 17 expression increases the amount of p62 (Fig. 4a). Since syntaxin 17 is required for the fusion between autophagosomes and lysosomes, it can be assumed that more viral particles were finally degraded by the autophagosomal-lysosomal pathway

scanning from three independent experiments. (i) (Left) Western blot analyses of cellular lysates derived from bafilomycin (BFLA)-treated HCV-negative cells (GND) and HCV-replicating cells (J6). (Right) Results of quantification by densitometric scanning from three independent experiments. (j) (Left) CLSM analyses of HCV-replicating cells (J6) that were treated with bafilomycin. Syntaxin 17 is visualized in red, and HCV core is visualized in green. The nuclei were stained with DAPI (blue). (Right) Results of quantification of the fluorescence intensity of syntaxin 17. Un., untreated. Magnifications, $\times 40$. *, $P < 0.05$; **, $P < 0.01$.

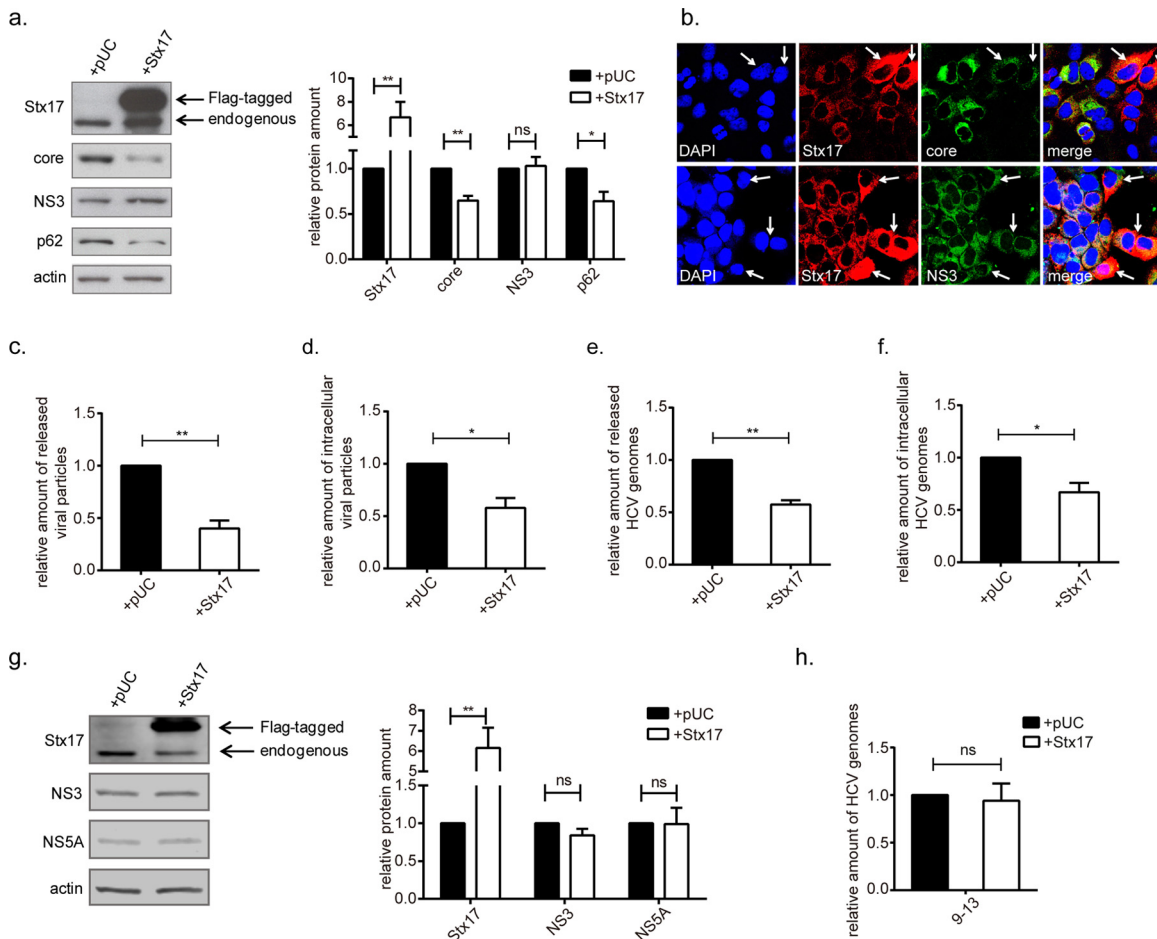


FIG 3 Overexpression of syntaxin 17 reduces the number of released HCV particles. (a) (Left) Western blot analyses of cellular lysates derived from HCV-replicating cells (J6) that were transfected with an expression vector carrying syntaxin 17 (pFlag-syntaxin 17) or a control vector (pUC19). Arrows, endogenous and overproduced syntaxin 17 (Flag tagged). (Right) Results of quantification by densitometric scanning from three independent experiments. (b) CLSM analyses of pFlag-syntaxin 17-transfected HCV-replicating cells (J6). HCV core (top) and NS3 (bottom) are visualized in green, and syntaxin 17 is visualized in red. The nuclei were stained with DAPI (blue). Arrows, syntaxin 17-overexpressing cells. Magnifications, $\times 100$. (c) The amount of infectious viral particles released into the supernatant derived from syntaxin 17-overexpressing or pUC19-transfected HCV-replicating cells (J6) was determined by a TCID₅₀ assay. The graph shows relative values from three independent experiments. (d) TCID₅₀ assay of intracellular infectious viral particles derived from syntaxin 17-transfected or pUC19-transfected HCV-replicating cells (J6). The graph shows relative values from three independent experiments. (e) The number of viral genomes in the supernatant derived from syntaxin 17-overexpressing or pUC19-transfected HCV-replicating cells (J6) was determined by RT-PCR. The graph shows relative values from three independent experiments. (f) RT-PCR analyses of intracellular viral genomes derived from syntaxin 17-transfected or pUC19-transfected HCV-replicating cells (J6). The graph shows relative values from three independent experiments. (g) (Left) Western blot analyses, using syntaxin 17-, NS3-, NS5A-, and actin-specific antisera, of cellular lysates derived from syntaxin 17-overexpressing or pUC19-transfected Huh9-13 cells carrying subgenomic replicons. Arrows, endogenous and overproduced syntaxin 17 (Flag tagged). (Right) Results of quantification by densitometric scanning from three independent experiments. (h) Quantification by RT-PCR of HCV-specific transcripts in syntaxin 17-overexpressing or pUC19-transfected Huh9-13 cells carrying subgenomic replicons. The graph shows relative values from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

when syntaxin 17 was overproduced, leading to the decreased number of released viral particles. Vice versa, inefficient fusion between autophagosomes and lysosomes caused by silencing of syntaxin 17 expression might shift the equilibrium between intracellular degradation and the release of viral particles toward release, resulting in elevated numbers of released infectious viral particles.

To experimentally control this, the formation of autolysosomes was inhibited by bafilomycin or autophagy was further induced by rapamycin in syntaxin 17-overproducing and pUC19-transfected HCV-replicating cells. Western blot analyses of the cellular lysates showed that bafilomycin treatment restored the intracellular level of core protein in syntaxin 17-overproducing

cells, while the induction of autophagy by rapamycin led to a further decrease in the intracellular amount of core protein when syntaxin 17 was overproduced (Fig. 5a). In accordance with these data, quantification of released viral particles by determination of the number of TCID₅₀s and quantification of the released viral genomes by RT-PCR show that the decreased viral release from syntaxin 17-overproducing cells can be rescued by bafilomycin treatment, which blocks the fusion between autophagosomes and lysosomes, while activation of autophagy by rapamycin leads to further decreased viral release (Fig. 5b and c). These data indicate that the decreased number of viral particles released from syntaxin 17-overexpressing cells is due to the facilitated formation of autolysosomes, in which HCV particles are degraded.

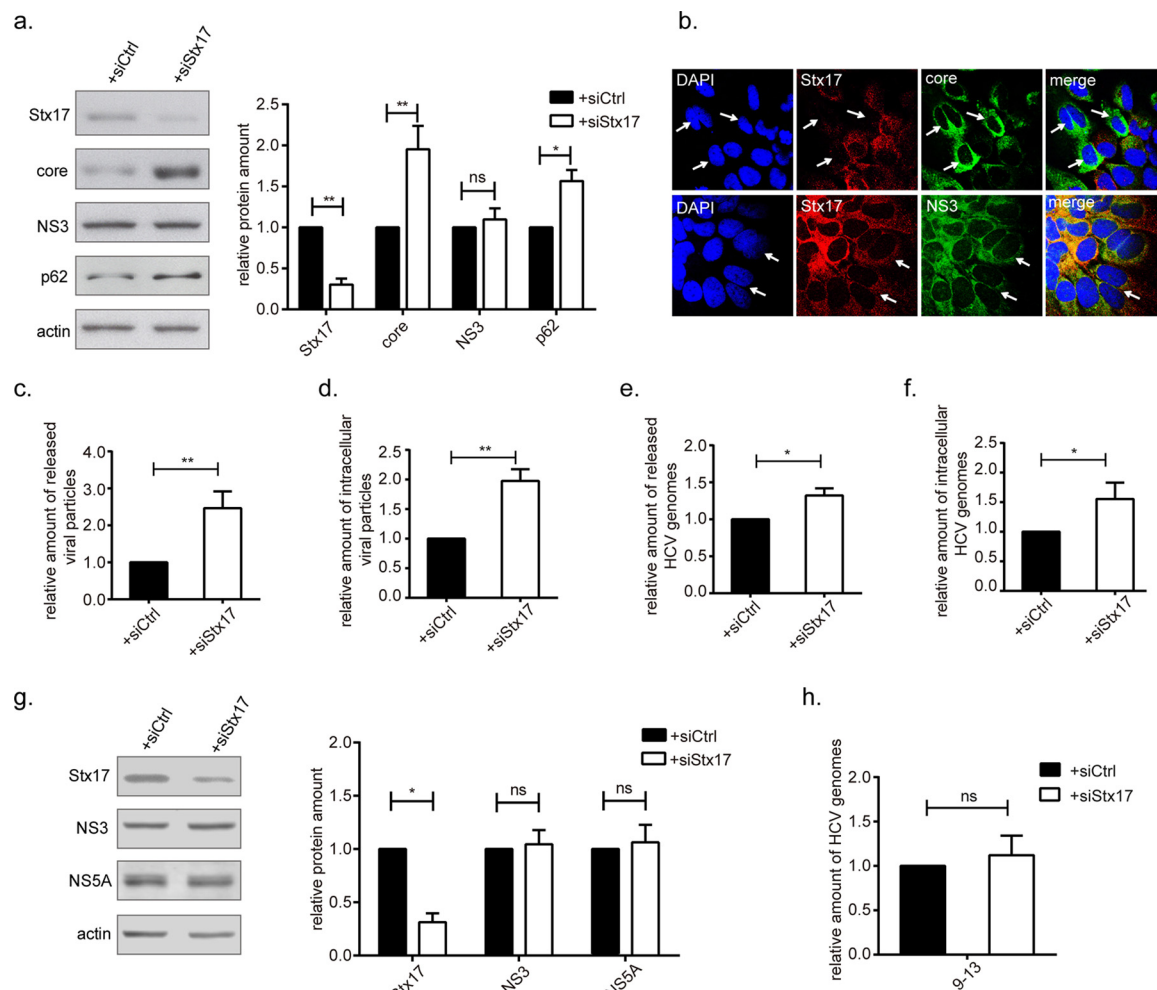


FIG 4 Silencing of syntaxin 17 expression facilitates viral particle release. (a) (Left) Western blot analyses of cellular lysates derived from HCV-replicating cells (J6) that were transfected with syntaxin 17-specific siRNA (siStx17) or control siRNA (siCtrl). Syntaxin 17-, NS3-, core-, p62-, and actin-specific antisera were used for detection. (Right) Results of quantification by densitometric scanning from three independent experiments. (b) CLSM analyses of syntaxin 17-specific siRNA-transfected HCV J6-positive cells. HCV core (top) and NS3 (bottom) are visualized in green, and syntaxin 17 is visualized in red. The nuclei were stained with DAPI (blue). Arrows, cells with smaller amounts of syntaxin 17. Magnifications, $\times 100$. (c) TCID₅₀ assay of infectious viral particles released into the supernatant derived from syntaxin 17-specific siRNA- or control siRNA-transfected HCV-replicating cells (J6). The graph shows relative values from three independent experiments. (d) TCID₅₀ assay of intracellular infectious viral particles derived from syntaxin 17-specific siRNA- or control siRNA-transfected HCV-replicating cells (J6). The graph shows relative values from three independent experiments. (e) RT-PCR analyses of viral genomes released in the supernatant derived from syntaxin 17-specific siRNA- or control siRNA-transfected HCV-replicating cells (J6). The graph shows relative values from three independent experiments. (f) RT-PCR analyses of intracellular viral genomes derived from syntaxin 17-specific siRNA- or control siRNA-transfected HCV-replicating cells (J6). The graph shows relative values from three independent experiments. (g) (Left) Western blot analyses of cellular lysates derived from syntaxin 17-specific siRNA- or control siRNA-transfected Huh9-13 cells carrying the subgenomic replicon. (Right) Results of quantification by densitometric scanning from three independent experiments. (h) Quantification by RT-PCR of HCV-specific transcripts in syntaxin 17-specific siRNA- or control siRNA-transfected Huh9-13 cells carrying the subgenomic replicon. The graph shows relative values from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

Western blot analyses of cellular lysates derived from HCV-replicating cells in which syntaxin 17 expression was silenced by specific siRNAs revealed an increased intracellular amount of core compared to the amount in untreated cells when the formation of autolysosomes was inhibited by bafilomycin or autophagy was induced by rapamycin (Fig. 5d). Quantification of released viral particles by determination of the number of TCID₅₀s shows that the increased release of viral particles after silencing of syntaxin 17 expression was further reinforced by induction of autophagy (rapamycin), while bafilomycin treatment did not have any significant effect (Fig. 5e). Due to the impaired expression of syn-

taxin 17 in syntaxin 17-specific siRNA (siStx17)-transfected cells, only a limited amount of syntaxin 17 was available to mediate the fusion with lysosomes and so represented the bottleneck. Therefore, the induction of autophagy by rapamycin does not end in fusion with lysosomes and the subsequent degradation of viral proteins, which were observed for the rapamycin-treated cells that were transfected with control siRNA. In the control transfected cells, the higher syntaxin 17 level enabled a more pronounced effect after treatment with rapamycin. In the case of siStx17-transfected cells, the impaired fusion of autophagosomes with lysosomes due to the lack of syntaxin 17 led to decreased lysosomal

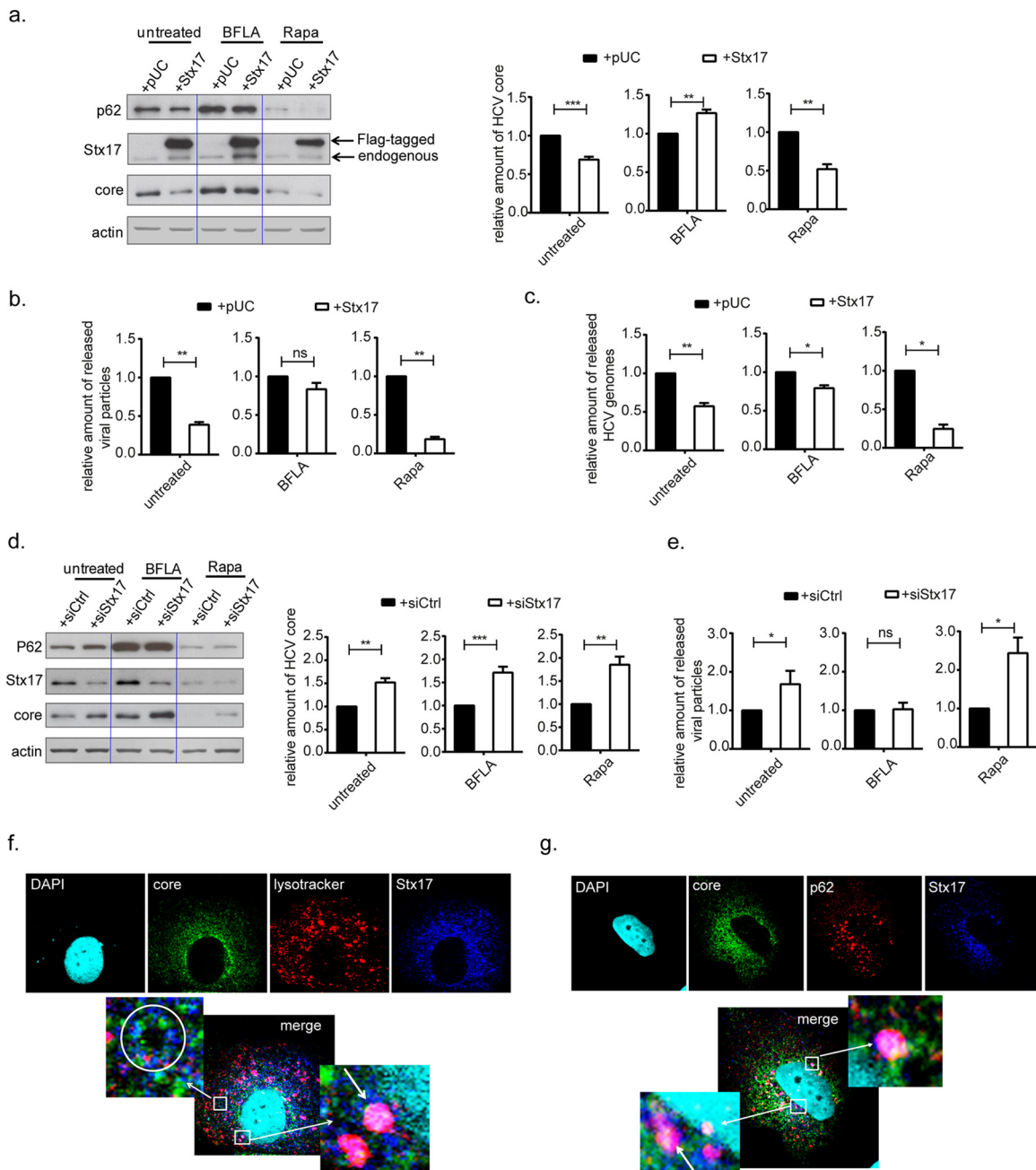


FIG 5 Syntaxin 17 controls the amount of released viral particles by affecting the formation of autolysosomes. (a) (Left) Western blot analyses, using syntaxin 17-, p62-, core-, and actin-specific antibodies, of cellular lysates derived from syntaxin 17- or pUC19-transfected HCV-replicating cells (J6) that were treated with bafilomycin (BFLA) or rapamycin (Rapa). (Right) Results of quantification by densitometric scanning from three independent experiments. In untreated cells, bafilomycin-treated cells, and rapamycin-treated cells, the particular amounts of core protein in syntaxin 17-transfected cells are always in reference to the amounts in the respective pUC19-transfected cells. (b) The amount of infectious viral particles in the supernatant derived from syntaxin 17-overexpressing or pUC19-transfected HCV-replicating cells (J6) that were treated with bafilomycin or rapamycin was determined by the TCID₅₀ assay. The graphs show relative values from three independent experiments. The values are in reference to those for the respective pUC19-transfected control. (c) The amount of viral genomes released in the supernatant derived from syntaxin 17-overexpressing or pUC19-transfected HCV-replicating cells (J6) that were treated with bafilomycin or rapamycin was determined by RT-PCR. The graphs show relative values from three independent experiments. The values are in reference to those for the respective pUC19-transfected control. (d) (Left) Western blot analyses, using syntaxin 17-, p62-, core-, and actin-specific antibodies, of cellular lysates derived from syntaxin 17-specific siRNA or control siRNA-transfected HCV-replicating cells (J6) that were treated with bafilomycin or rapamycin. (Right) Results of quantification by densitometric scanning from three independent experiments. The values are in reference to those for the respective control siRNA (siCtrl). (e) Quantification of infectious viral particles in the supernatant derived from syntaxin 17-specific siRNA or control siRNA-transfected HCV-replicating cells (J6) that were treated with bafilomycin or rapamycin by the TCID₅₀ assay. The graphs show relative values from three independent experiments. (f) CLSM analyses of HCV-replicating cells (J6). HCV core is visualized in green, and syntaxin 17 is visualized in blue. Lysosomes were stained with LysoTracker (red). The nuclei were stained with DAPI (turquoise). Higher magnifications of the boxed areas are shown at the bottom. Magnifications, $\times 100$. (g) CLSM analyses of HCV-replicating cells (J6). HCV core is visualized in green, p62 is visualized in red, and syntaxin 17 is visualized in blue. The nuclei were stained with DAPI (turquoise). Magnifications, $\times 100$. Higher magnifications of the boxed areas are shown at the bottom. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

degradation, which was already so moderate that no significant effect was detectable by an additional inhibition of lysosomal acidification by bafilomycin. These data suggest that the silencing of syntaxin 17 expression leads to impaired autophagosome-lysosome fusion and thereby shifts the equilibrium between release and lysosomal degradation toward an increased release of infectious viral particles.

The data described above indicate a functional interaction between the autophagosomal SNARE protein syntaxin 17 and the HCV life cycle. To study whether core can be found within autophagosomal-lysosomal structures characterized by the presence of syntaxin 17, confocal immunofluorescence microscopy analyses of HCV-replicating cells were performed (Fig. 5f). Syntaxin 17-specific (blue) and core-specific (green) antisera were used. Lysosomes were stained by LysoTracker (red). The CLSM analyses showed that, indeed, a fraction of core (green) can be found within vesicular structures stained in their interior by LysoTracker (red) and on their surface in a ring-like structure by syntaxin 17-specific antiserum (blue) (Fig. 5f). This indicates that a fraction of core protein is found in autolysosomes. This could be confirmed by CLSM analyses using the autophagy marker p62 (Fig. 5g).

Taken together, these data demonstrate that in HCV-replicating cells, syntaxin 17 is an essential factor controlling the equilibrium between the release of viral particles from the autophagosomal pathway and the degradation in lysosomes.

DISCUSSION

Autophagy has been described to be involved in different steps of the HCV life cycle. The interaction between HCV NS proteins and factors of the autophagosomal system plays an important role for the formation of the membranous web and for the reorganization of intracellular membranes. NS5A was described to induce autophagy by triggering the fusion of autophagosomes with lysosomes (39). In accordance with this, NS5A, NS5B, and viral RNA were found on autophagosomal structures (40). Moreover, HCV induces autophagy via activation of the unfolded protein response (UPR) (41, 42). With respect to the release of HCV, it was found that silencing of Beclin-1 and Atg7 leads to an impaired release of infectious viral particles (24). In a recent report, it was found that TIP47 is required for the release of HCV (14, 15). However, if the Rab9-binding domain of TIP47 is destroyed, TIP47 preserves the capacity to bind to HCV particles, but the complex is targeted to the autophagolysosome and prevented from being released (15).

Here we identify syntaxin 17 to be a novel factor involved in the life cycle of HCV. Syntaxin 17 is an autophagosomal SNARE protein, and it is required for the fusion between autophagosomes and endosomes/lysosomes at a late step of autophagy. We found that in HCV-replicating cells, the amount of syntaxin 17 is reduced due to impaired expression and increased turnover. The decreased amount of syntaxin 17 did not depend on virus morphogenesis, as shown by analyses of subgenomic replicons. In both systems, in the full-length replicon and in the subgenomic replicon, NS5A was described to bind c-Raf and thereby to withdraw c-Raf from the MEK/ERK signaling cascade, which leads to impaired MEK/ERK signaling, which is involved in the control of syntaxin 17 expression (6–8).

Overexpression of syntaxin 17 leads to a reduced amount of released viral particles and HCV genomes. Elevated levels of syntaxin 17 promote autophagosome-lysosome fusion and thereby facilitate the targeting of intracellular particles/genomes from the

autophagosome to the autophagolysosome followed by their degradation, leading to a decreased amount of intracellular particles/genomes. The decreased amount of viral particles/genomes released from syntaxin 17-overexpressing cells is not due to interference with genome replication, as shown by the unaffected level of the NS protein NS3. This was further confirmed by the use of subgenomic replicons that enable the analyses of genome replication in the absence of virus morphogenesis; here, no effect on replication was observed. In accordance with this, the decreased release of viral particles from syntaxin 17-overexpressing cells could be rescued by inhibition of lysosomal acidification with bafilomycin, indicating that, indeed, the function of syntaxin 17 involved in autophagosome-lysosome fusion affects the HCV life cycle. Vice versa, silencing of syntaxin 17 expression resulted in an elevated amount of released viral particles due to the impaired autophagosome-lysosome fusion, while replication was not significantly affected, as demonstrated by the use of subgenomic replicons.

The viral life cycle is characterized by the fact that the number of viral particles formed is much greater than the number of infectious particles released. It is assumed that an infected hepatocyte produces about 1,000 virions per day but only 50 are released, raising the question about the fate of the remaining 950 virions (16, 17). Moreover, all structural proteins are formed on the basis of a polyprotein. This means that for each structural protein produced, an equivalent amount of nonstructural proteins is formed, which necessitates the removal of these proteins.

These functions could be fulfilled in part by the autophagosomal/autolysosomal system. However, induction of autophagy does not automatically mean that the process ends for all components in the autolysosome/lysosome. Syntaxin 17 might represent a trigger that enables a tight control of this process at a late point, affecting the number of viral particles that are finally released from the cell, i.e., in exosomes, or that are degraded in the lysosomal compartment (38).

Our data demonstrate that a larger amount of syntaxin 17 prevents the release of HCV by facilitating autophagosome-lysosome fusion. In accordance with this, in HCV-replicating cells a reduced amount of syntaxin 17 was found, ensuring that the equilibrium between released and lysosomally trapped/degraded virions is not completely shifted toward degradation. This is achieved by an impaired expression of syntaxin 17 in HCV-replicating cells and by a self-controlled loop: induction of autophagy leads to a decreased amount of syntaxin 17, preventing an induction of lysosomal degradation that is too strong.

In light of the observation that syntaxin 17 represents a novel factor controlling the release of viral particles, syntaxin 17 could be a target for the treatment of chronic HCV infections. By prevention of the release and induction of intracellular degradation of viral proteins, increased antigen processing and an increased presentation of HCV-specific antigens could be achieved, facilitating the recognition and elimination of HCV-replicating cells by the cellular immune response.

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